Published in final edited form as:

Dev Biol. 2017 February 01; 422(1): 47–57. doi:10.1016/j.ydbio.2016.12.004.

Tissue specific regulation of the chick Sox10E1 enhancer by different Sox family members

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Abstract

The transcription factor Sox10 is a key regulator of vertebrate neural crest development and serves crucial functions in the differentiation of multiple neural crest lineages. In the chick neural crest, two cis-regulatory elements have been identified that mediate *Sox10* expression: Sox10E2, which initiates expression in cranial neural crest; Sox10E1 driving expression in vagal and trunk neural crest. Both also mediate *Sox10* expression in the otic placode. Here, we have dissected and analyzed the Sox10E1 enhancer element to identify upstream regulatory inputs. Via mutational analysis, we found two critical Sox sites with differential impact on trunk versus otic Sox10E1 mediated reporter expression. Mutation of a combined SoxD/E motif was sufficient to completely abolish neural crest but not ear enhancer activity. However, mutation of both the SoxD/E and another SoxE site eliminated otic Sox10E1 expression. Loss-of-function experiments reveal Sox5 and Sox8 as critical inputs for trunk neural crest enhancer activity, but only Sox8 for its activity in the ear. Finally, we show by ChIP and co-immunoprecipitation that Sox5 directly binds to the SoxD/E site, and that it can interact with Sox8, further supporting their combinatorial role in activation of Sox10E1 in the trunk neural crest. The results reveal important tissue-specific inputs into *Sox10* expression in the developing embryo.

Keywords

chick; Sox10; neural crest; otic

Introduction

Neural crest cells are multipotent stem-like cells unique to vertebrate embryos. They initially arise within the dorsal aspect of the neural tube, but subsequently undergo an epithelial to mesenchymal transition and migrate extensively throughout the embryo. Upon reaching their final destinations, they differentiate into a wide range of derivatives, including neurons and glia of the peripheral nervous system, craniofacial cartilage and bone, and pigment cells of the skin.

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A multilevel gene regulatory network, integrating environmental cues and transcriptional inputs, underlies sequential steps in neural crest formation (Meulemans and Bronner-Fraser, 2004, Sauka-Spengler and Bronner-Fraser, 2008a, Green et al., 2015). This culminates in expression of a set of "neural crest specifier genes" like *FoxD3* and *SoxE* genes (Meulemans and Bronner-Fraser, 2004, Sauka-Spengler and Bronner-Fraser, 2008b, Simoes-Costa et al., 2014, Simoes-Costa et al., 2015) that reflect formation of bona fide neural crest cells. The combined action of these transcription factors induces downstream effector genes that regulate migration and subsequent cell fate choice. Despite species-specific differences, the main components of this regulatory network are highly conserved across vertebrates (Green et al., 2015; Haldin and LaBonne, 2010).

Despite this deep conservation across species, there are important differences between neural crest subpopulations along the body axis. For example, only cranial but not trunk neural crest cells contribute to cartilaginous elements of the face (Le Douarin, 1980; Lwigale et al., 2004; Simoes-Costa and Bronner, 2016). Interestingly, these differences in developmental potential are mirrored by the existence of different regulatory elements that control neural crest gene expression at different axial levels. As case in point, despite the fact that neural crest specifier genes *FoxD3* and *Sox10* are expressed in neural crest cells along the entire body axis, different cis-regulatory elements mediate their expression in the head than in the trunk (Betancur et al., 2010; 2011, Simoes-Costa et al., 2012; Simoes-Costa and Bronner, 2016). For example, two cis-regulatory elements, Sox10E2 and Sox10E1, mediate *Sox10* expression in the head versus the trunk, respectively.

The cranial Sox10E2 enhancer has been well characterized (Betancur et al., 2010) and mediates initial expression in the cranial neural crest at Hamburger Hamilton (HH) stage 9 via direct inputs from Sox9, Ets1, and cMyb (Betancur et al., 2010). This same enhancer also drives expression in the otic placode, requiring the same transcription factor binding sites but via inputs from paralogous factors, Sox8 and Pea3 in combination with cMyb (Betancur et al., 2011). Another enhancer, Sox10E1, drives *Sox10* expression in migrating neural crest cells at vagal and trunk levels, as well as the otic placode. However, its regulatory inputs have not been previously explored.

Here, we examine the mechanisms controlling Sox10E1 activity, in search of upstream regulators controlling its trunk neural crest as well as otic activity. By generating serial deletions and mutating potential binding sites, we find that two SoxD/E sites are required for enhancer activity in both the trunk neural crest and developing ear. While mutation of the SoxD/E site alone abolishes trunk enhancer activity, additional mutation of a second SoxE site is needed to eliminate otic expression. Knockdown experiments identified Sox5 and Sox8 as upstream regulators of Sox10E1. While Sox8 is important for activation in both otic and trunk neural crest, Sox5 is only involved in trunk neural crest enhancer activation. We further show that Sox5 and Sox8 can heterodimerize and that Sox5 is specifically recruited to the identified SoxD/E site in the Sox10E1 enhancer. Taken together, our results suggest that Sox5 and Sox8 cooperate in regulating Sox10E1 in the trunk neural crest.

Results

Sox10E1 is active in migrating vagal and trunk neural crest and otic placode

To analyze the spatiotemporal activity of the Sox10E1 enhancer, we electroporated chick embryos at different developmental time points with a GFP reporter under the control of the enhancer fragment. As previously reported (Betancur et al, 2010), Sox10E1 starts to activate GFP expression in migrating neural crest at vagal/anterior trunk levels around HH15. GFP expression is not detected in the posterior trunk until HH18, when segmental migratory streams of GFP positive cells are visible (Fig. 1A, 1C), reflecting endogenous Sox10 expression at this stage (Fig. 1B–E). Although *Sox10* mRNA and protein are present in migrating neural crest (Fig. 1B, D), enhancer-driven expression is not detectable in early delaminating neural crest cells, suggesting the presence of additional, yet unidentified regulatory elements that initiate Sox10 expression in this region. In addition to vagal/trunk neural crest cells, Sox10E1 activates GFP in the otic vesicle as it begins to invaginate around HH12-13 and onward (Fig. 1G). Reporter expression is never observed in cranial neural crest cells (Fig. 1F–G).

Identification of essential regions within the Sox10E1 enhancer

The previously isolated Sox10E1 fragment (Betancur et al., 2010) is 620bp in length (Fig. 2A). To determine essential regions mediating Sox10E1 activity, we performed a series of deletions and mutations. Initially, we created deletions to sequentially minimize the region in approximately 100bp steps from each side. We then tested those constructs for their ability to drive GFP expression *in ovo* (summarized in Fig. 2B). Two fragments lacking 120bp or 234bp on the 5′ site (Δ1 and Δ2) were still able to activate GFP in migratory trunk neural crest cells (Fig. 3A; 10/10) as well as the otic placode (Fig. 3B; 10/10). Interestingly, one other fragment of 260bp length (Δ8), lost activity in migratory neural crest cells (Fig. 3C), but was still strongly active in the otic vesicle (Fig. 3C–D, 10/10 each). Another fragment termed Δ9, where the sequence stretch 5′ of Δ8 was extended until the beginning of Δ2 still retained strong GFP expression in the trunk (summarized in Fig. 2B). All other deletions completely lost activity (Fig. 2B).

Mutation of Sox10E1 binding sites to identify potential inputs

We next searched the Sox10E1 sequence for putative transcription factor binding sites using the Jaspar and Transfac databases. Predicted sites were analyzed for their conservation across species using the UCSC and ECR genome browsers (Fig. 2A). Notably, most of the conserved binding sites fall within the remaining sequence present in Sox10E1Δ8, the fragment retaining otic expression but loosing trunk activity (Fig. 2A–B). We selected nine sites and mutated them by substituting 6–15 bp of the core binding site with eGFP coding sequence (Fig. 2C). We chose these sites either because of high sequence conservation or because the putative transcription factors are well known as regulatory factors implicated in neural crest development. We mutated two Sox and two FoxD3 binding sites, as well as individual sites for Myb, Ets and Zic. Furthermore, we mutated a highly conserved site predicted to bind stem cell factor Arid3A.

Mutation of one potential SoxD/E binding site (mutant M4) completely abolishes activity in the migratory trunk neural crest but has no effect on ear expression (Fig. 4A–C, 5/5 each). All other mutations tested were still able to drive GFP expression in the neural crest (summarized in Table 1). While mutation of SoxD/E in construct M4 completely abolishes trunk crest enhancer activity, mutation of another SoxE site present in the Sox10E1 fragment (mutant M6) has no effect on trunk neural crest expression (Fig. 4D, 5/5). Interestingly, GFP expression in the otic placode of the M6 mutant seemed weaker compared to expression of the M4 construct (Fig. 4E–F, 3/5). When we quantified the fluorescence intensity of the SoxE (M6) and SoxD/E (M4) mutants in the otic vesicle, we indeed observed a 50% reduction in fluorescence intensity of M6 compared to M4 (Fig. 4J). When both sites are mutated, both otic and trunk activity are lost (Fig. 4G–I, 5/5 each). As shown in Fig. 2B–C, both sites fall within the sequence stretch retained in Sox10E1Δ8.

Sox5 and Sox8 are expressed in the neural crest, but only Sox8 is in the otic vesicle

The results of our mutational analysis suggest that SoxD and SoxE proteins may serve as potential regulators of Sox10E1 activity. A potential candidate for binding to the M4 site is the SoxD family member, Sox5, previously implicated in cranial neural crest formation in chick and Xenopus (Perez-Alcala et al., 2004, Nordin and LaBonne, 2014). Consistent with previous reports (Perez-Alcala et al., 2004), we observed *Sox5* expression as early as stage HH7 in the forming cranial neural folds and its expression becomes more pronounced when neural crest cells arise at stage HH9-10. Though stronger in the neural crest, it is also expressed in the neural tube but absent from the ectoderm and not expressed in the otic placode or the otic vesicle (Fig. 5A). *Sox5* is expressed in the migratory neural crest cells at vagal and trunk levels (Fig. 5A–B) making it a potential candidate as a regulator of Sox10E1 in this population. It also is strongly expressed in the neural crest streams coming from R4 and R6, which surround the otic vesicles (Fig. 5A), with lower expression in the surrounding mesoderm.

All SoxE family members are expressed during chick neural crest development, with expression of *Sox8* and *Sox9* both preceding *Sox10* and thus potentially able to induce its expression. *Sox8* transcripts are present in otic-epibranchial precursor cells at stage HH8-, and its expression persists throughout the time of otic vesicle formation. Sox8 activates endogenous Sox10 expression in this region through regulation of the Sox10E2 enhancer (Betancur et al., 2011). However, it continues to be strongly expressed during formation of the otic placode and the otic vesicle later (Fig. 5C), by which time the Sox10E1 enhancer becomes activated. As previously shown (McKeown et al., 2005), *Sox8* is also transiently expressed in early migrating trunk neural crest cells (Fig. 5D), and thus overlaps with *Sox5* at relevant stages of trunk Sox10E1 activation.

Loss of function experiments identify Sox5 and Sox8 as inputs into Sox10E1

To test for a potential regulatory role, we performed loss-of-function experiments with individual Sox family members using morpholino antisense oligomers. To this end, we concomitantly electroporated the full length Sox10E1 enhancer construct with individual morpholinos and analyzed subsequent enhancer mediated reporter expression. Morpholinos to Sox8, 9 and 10 are well characterized (Betancur et al., 2010, 2011, Barembaum and

Bronner, 2013, Simoes-Costa and Bronner, 2016). To verify efficiency of knockdown by the Sox5 morpholino, we examined its effects on Sox5 protein expression compared with control morpholino by Western blot analysis. For this purpose, we performed stage HH4 electroporations to completely abolish Sox5 expression. The results confirm that Sox5 MO indeed causes loss of Sox5 protein in electroporated embryos (Fig. 6A).

Electroporation of a control morpholino does not affect Sox10E1 driven mCherry expression (0/19) in the trunk (Fig. 6B). In contrast, co-electroporation of a morpholino targeting Sox5 significantly reduces Sox10E1 reporter expression in trunk neural crest (Fig. 6C), with 14 out of 15 embryos showing reduced or no mCherry expression in the presence of the morpholino. When we quantify the fluorescence intensity, Sox10E1 reporter expression upon Sox5 knockdown is reduced to an average of 27% of the level measured in control morpholino experiments (Fig. 6F). Despite the strong effect on enhancer activity, endogenous Sox10 levels are only mildly affected by Sox5 reduction (Fig. S1A–F, 3/10). To further dissect which SoxE family members are involved in regulating Sox10E1 activity, we individually knocked down Sox8, 9 and 10. A striking reduction in trunk reporter expression is observed upon knockdown of Sox8 (Fig. 6D, 13/15), similar to the effect of Sox5 knockdown. We did not observe any reduction of endogenous Sox8 levels when Sox5 was depleted (Fig. S1G–H, 0/5). Knockdown of Sox9 does not affect Sox10E1 expression (0/8, quantified in Fig. 6F). However, the presence of a Sox10 morpholino reduces enhancer activity in a subset of the embryos (7/16, Fig. 6E), suggesting that once activated, Sox10 itself might participate in regulating its own expression levels. Because we had identified a Zic binding site in the critical region mediating trunk enhancer activity, we further tested the effect of knocking down Zic1. Similar to Sox9 knockdown experiments, the Sox10E1 reporter was not affected by the presence of the Zic1 morpholino (quantified in Fig. 6F). This is in line with the results we obtained with the M3 mutant that is missing a functional Zic binding site, which also did not show any reduction in reporter expression (10/10, data not shown).

Knocking down Sox8 also affects otic reporter expression. While Sox10E1 driven expression is slightly weaker than in control experiments (Fig. 7A–A′) when electroporations are performed at stage HH8 or earlier (Fig. 7B–B′), its expression is largely unaffected when electroporations are carried out at stage HH9 (Fig. 7C–C′). A similar effect was noted when knocking down Sox9 specifically in the ectoderm. Knockdown of Sox9 at early stages diminished Sox10E1 reporter expression in some cases (5/18, Fig. 7D–D′), though the percentage of embryos showing this phenotype was higher for Sox8 (10/18). Only 2 out of 10 embryos electroporated with the Sox10 morpholino exhibit reduced expression in the otic, suggesting little or no effect. Sox5 morpholino does not affect activation of the Sox10E1 reporter expression in the otic vesicle at any stage (1/10, Fig. 7E–E′). These results are in line with our observations that Sox5 is not expressed in the otic vesicle itself (Fig. 5A) and mutation in the SoxD/E binding site does not affect otic Sox10E1 activity (Fig. 4B–C). Quantification of fluorescence intensities in the ear was not feasible due to spherical aberration and autofluorescence of the epithelial sphere. Taken together, the data suggest that Sox8 regulates Sox10E1 expression in the ear, though there might be some redundancy with other SoxE family members. Furthermore, our results suggest that

additional elements may be involved in activating Sox10E1 in the otic vesicle, given the rather mild effects of our knockdown experiments.

Co-immunoprecipitation suggests an interaction between Sox5 and Sox8

To test for a potential interaction between Sox5 and Sox8, we performed immunoprecipitation experiments. Because antibodies that are specific to avian Sox8 are not available, we electroporated chick embryos with a construct expressing a FLAG tagged version of chicken Sox8. After immunoprecipitation with an antibody to Sox5 or FLAG, we tested for interaction of Sox8 and Sox5 using either an anti-FLAG or anti Sox5 antibody. In both cases, we were able to pull down the opposite protein from lysates of embryos electroporated at stage HH11 (Fig. 6G), indicating that Sox5 and Sox8 can interact with each other.

Direct binding of Sox5 to the SoxD/E site

The results of our mutation and knockdown studies indicate that the identified SoxD/E binding site is critical for Sox10E1 enhancer activity in the trunk, potentially involving direct binding of Sox5 and Sox8 to this region. In order to test this hypothesis we performed chromatin immunoprecipitation (ChIP) experiments on dissected trunk neural tubes of wild type chick embryos. Using a ChIP grade antibody to Sox5, we found that Sox5 directly bound to the Sox10E1 enhancer (Fig. 6H). Interestingly, Sox5 levels are highest at the M4 site alone, and less pronounced across the M4 and M6 sites together. Furthermore, only low levels of Sox5 are found at the 5′ region of the Sox10E1 enhancer, outside of the M4 binding site. Taken together, these results suggest that Sox5 is specifically recruited to the M4 binding site, and this is responsible for activating Sox10E1 in the trunk neural crest.

Discussion

Sry-related high mobility (HMG)-box Sox transcription factors serve widespread functions during development and are found throughout the animal kingdom. This large family is comprised of subgroups A-H, which all possess a highly homologous HMG-type DNA binding domain but share little overall homology outside this region (Kamachi and Kondoh, 2013, Bowles et al., 2000). Subgroup SoxE, comprised of Sox8, 9 and 10, is associated with neural crest cell identity in all vertebrate species examined. In chick embryos, expression of *Sox8* and *Sox9* precedes *Sox10*, which is first evident in delaminating/early migrating neural crest cells along the entire extent of the body axis and is a critical upstream regulator of multiple neural crest lineages (Green et al., 2015). Later, *Sox10* expression is maintained in neuronal, glial and melanocytic lineages. Furthermore, it has recently been shown that Sox10 alone is sufficient to reprogram fibroblasts to a neural crest fate, highlighting the importance of this factor during neural crest specification (Kim et al., 2014).

Sox5 is the only SoxD member expressed in neural crest cells. In the chick, *Sox5* is expressed early on in premigratory neural crest in the head region. Sox5 over-expression promotes neural crest formation and increases expression of *FoxD3* and *Sox10* (Perez-Alcala et al, 2004). It seems to do so by directly activating expression of RhoB in transfected cells, which in turn induces *Sox10* expression. Here we show that Sox5 is recruited to

binding sites in the Sox10E1 enhancer, also demonstrating a direct regulatory interaction between Sox5 and Sox10. In the trunk, however, this interaction serves more to maintain rather than induce initial endogenous *Sox10* expression. Consistent with this, we observed a rather modest decrease in endogenous *Sox10* levels upon Sox5 knockdown. In most cases, Sox10 still continues to be robustly expressed whereas enhancer expression in dramatically reduced. However, endogenous Sox10 expression is already observed before Sox10E1 activation (Figure 1), suggesting that other elements, not explored in this study, initiate trunk Sox10 expression. We hypothesize that once Sox10 is activated, its expression is driven by several regulatory elements, including but not exclusive to Sox10E1, that cooperate in fine-tuning and subsequent lineage specific diversification among subpopulations.

In addition to the neural crest, SoxE and SoxD family members are expressed in the developing ear. Most components of the ear are derived from the otic placode, a region of thickened ectoderm that forms adjacent to the hindbrain and subsequently invaginates to form the otic vesicle. Via complex morphogenetic rearrangements and patterning events, this vesicle is transformed into the entire inner ear (reviewed in Chen and Streit, 2013).

To gain insight into the regulatory interactions important for neural crest and ear development, we have dissected the cis-regulatory inputs necessary for expression mediated by the Sox10E1 enhancer element. Similar to the cranial Sox10E2 enhancer, the vagal/trunk Sox10E1 enhancers drives expression in both the neural crest and otic placode. Notably, the combined expression pattern driven by Sox10E1 and E2 largely resembles most of that of endogenous *Sox10* expression. The exceptions are that neither of these elements is active in delaminating trunk neural crest cells, despite the fact that *Sox10* transcripts are already present there. Although some of these differences may be due to time required for folding the GFP reporter protein, this cannot account for the large temporal delay in expression mediated by Sox10E1 in migrating trunk neural crest cells. Thus, this suggests that there may be additional regulatory elements that control early endogenous *Sox10* expression. In particular, endogenous *Sox10* must be activated in delaminating trunk neural crest cells before Sox10E1 becomes active and maintains *Sox10* expression during migration.

Notably, multiple regulatory elements with overlapping spatiotemporal activities have been identified for mouse Sox10, with one of them sharing sequence similarity to the chicken Sox10E1 element (Werner et al., 2007). Interestingly, those enhancer elements further bear identical transcription factor binding sites, suggesting the same regulators are involved in controlling their activity (Wahlbuhl et al., 2012). Thus, *Sox10* expression in a particular tissue may reflect the combined activity of several regulatory elements rather than a single element contributing a highly specific spatial or temporal aspect to the overall activity. This may represent a valuable fail-safe mechanism that accounts for the high levels and robustness of *Sox10* expression during embryonic development.

In the chick, Sox10E1 and Sox10E2 are both active at certain stages during otic development and also share common transcription factor binding sites. Hence, the robust expression in the developing ear and the mild effects of our knockdowns in this area might reflect redundant regulatory activity of those elements. Furthermore, a third enhancer termed

L8 has been described that shows similar but weaker activity to Sox10E1 in the ear (Betancur et al., 2011).

By deletion and mutational analysis, we identified two critical Sox binding sites important for Sox10E1 mediated activity in both tissues. While one of these (M4) is putatively bound by SoxD and/or SoxE proteins, the other one (M6) is thought to be recognized by SoxE proteins only. Interestingly, mutation of the M4 motif alone abolishes enhancer activity in the trunk crest while leaving otic expression intact. On the other hand, mutation of the M6 motif reduces otic expression, while having no effect on trunk crest expression. Combined mutation of both elements eliminates activity completely, demonstrating their importance in both tissues. The tissue specific differences are likely due to tissue specific recruitment of binding partners, e.g. different Sox family members. We show that Sox5 is indeed recruited to the M4 site in migrating trunk neural crest cells, where it is abundantly present. In contrast, Sox5 is not expressed in otic placode cells. Accordingly, the activity of the Sox10E1 enhancer is significantly reduced in the presence of a Sox5 morpholino in the trunk, but not in the ear. During otic development, other SoxD family members (e.g. Sox13) could utilize this site to activate Sox10E1. Alternatively, a SoxE family member (e.g. Sox8) might be recruited to this site instead. In any case, binding to this site is not crucial for enhancer function in the otic placode. Instead, the other Sox site identified in this study (M6) seems to be most critical for otic expression, as mutating this site reduces the intensity of the enhancer driven expression. However, mutations in both M4 and M6 result in complete loss of activity, suggesting that both sites are necessary for full functionality.

It is interesting to note that fragment Sox10E1Δ8 looses trunk neural crest activity despite containing both Sox sites. One possibility is that additional critical binding sites are present outside of Sox10E1Δ8. Another possible explanation for this discrepancy could lie in the DNA binding nature of Sox proteins. Besides their transactivation activity, Sox proteins have been hypothesized to function as architectural proteins that are involved in three-dimensional shaping of promoters and associated DNA binding proteins (Chew and Gallo, 2009). In particular, group D members are thought to act in this manner, as they are missing transactivation domains. As the M4 site lies very close to the 5′ end of the Sox10E1Δ8 fragment, those structural features could be impaired even when Sox5 binding is still possible.

Loss of function of Sox8 reduces expression mediated by the Sox10E1 enhancer in trunk neural crest, similar to a knockdown of Sox5. Furthermore, our immunoprecipitation experiments demonstrated that Sox5 and Sox8 can physically interact. This is consistent with a scenario in which Sox5 and 8 cooperatively bind to the M4 motif to activate Sox10E1 in trunk neural crest. Such an interaction between SoxD and SoxE protein family members has been described during chondrogenesis. In mice, Sox5, Sox6 and Sox9 cooperate in activation of Col2A1 transcription. All three proteins bind the same 48bp enhancer element within the gene and also cooperatively can activate transcription in non-chondrogenic cells (Lefebvre et al., 1998). Experiments in human chondrogenic cells have further shown that Sox8 can replace Sox9 in activating Col2A1 in combination with Sox5 and Sox6 (Herlofsen et al., 2014), though at reduced efficiency. These studies, however, did not address if this involves a direct interaction between Sox5/6 and Sox8/9. A more recent study addressing the

potential of SoxE proteins to interact with other Sox family members suggests that they indeed are capable of heterodimerization, although homodimerization is favored (Huang et al., 2015). Consistent with these studies, we show that Sox5 and Sox8 are able to bind to each other *in vitro*. The fact that Sox5 is bound to the M4 site, together with the fact that knockdowns of either Sox5 or Sox8 show a similar penetrance of phenotype, further supports the idea that both factors are required cooperatively to activate Sox10E1 in the trunk. As Sox5 lacks a transactivation domain, it must recruit other transcription factors that induce Sox10E1 activity. It remains to be elucidated if Sox5 and Sox8 bind the SoxD/E site individually, or if Sox8 is indirectly recruited to the site via Sox5. We did not detect any reduction in Sox8 expression after Sox5 knockdown, suggesting that Sox5 does not regulate Sox8 expression (Fig. S1G–H).

Of all factors tested, only Sox8 knockdown reduced Sox10E1 reporter expression in the otic vesicle. The effect was, however, stage sensitive and only present in embryos electroporated at stage HH8 but not at stage HH9. One possible explanation is that this effect is indirect via loss of Sox8 affecting early expression of Sox10 via a different enhancer. Our previous studies have shown that Sox10E2 driven expression in the otic placode is activated by Sox8, together with Pea3 and cMyb beginning around stage HH9+. Knockdown of Sox8 alone strongly reduces onset of endogenous Sox10 expression in the ear (Betancur et al., 2011). Once Sox10 is activated, it may regulate its own expression via an autoregulatory feedback loop, similar to what has been shown to occur in the context of neural crest *Sox10* expression in mice (Wahlbuhl et al., 2012).

In summary, we have identified two critical Sox binding sites in the chicken Sox10E1 enhancer, which differentially control activity in the trunk neural crest and the otic vesicle. Furthermore, Sox5 binds this enhancer in trunk neural crest and can heterodimerize with Sox8, both of which are critical regulators of Sox10E1 enhancer activity in the trunk neural crest. While Sox5 is restricted to the neural crest, Sox8 also seems to be involved in regulation of otic Sox10E1 activity. However, Sox10E1 in the otic vesicle is likely to utilize additional yet to be identified inputs. Similar to what was observed for the Sox10E2 enhancer, our study shows that the same Sox10 enhancer can be activated by different inputs in different tissues. Thus, tissue specific expression occurs via the combined availability of open regulatory regions within the genome together with the composition of regulatory factors present in particular tissues.

Material and Methods

Embryos

Fertilized chicken eggs were obtained from commercial sources and incubated at 37°C until embryos reached the desired developmental stages. Electroporated and wild type embryos were collected in Ringer's and staged according to the criteria of Hamburger and Hamilton (Hamburger and Hamilton, 1992)

Electroporation of chick embryos

Embryos were electroporated as previously described (Sauka-Spengler and Barembaum, 2008). DNA was injected at 2. DNA concentration. When coelectroporating morpholinos, 1mM final concentration was used in all experiments, except stated otherwise. Stage HH4 embryos were explanted on filter paper and injected underneath the blastoderm. Electroporation was carried out in a modified electroporation chamber and 5pulses of 5.2V were applied for 50ms. Stage HH11 embryos were injected *in ovo* into the trunk neural tube lumen. Platinum wire electrodes were placed on either side of the embryo and 3 pulses of 20V, 30ms, were applied to deliver the DNA into the cells. To target the otic vesicle, DNA was placed above the embryo at future midbrain levels in stage HH8-9 embryos. Electrodes were placed with the anode underneath the embryo and the cathode above the vitelline membrane and charges were delivered in 3 pulses of 9V, 30ms. Eggs were sealed and reincubated until the desired developmental stage for further processing.

Morpholino experiments

Knockdown experiments were performed with translation-blocking morpholino antisense nucleotides (Gene Tools). All morpholinos used were FITC tagged at the 3′ end and used at 1mM final concentration. The sequences were as follows: Control: 5′??-ATGGCCTCGGAGCTGGAGAGCCTCA-3′; Sox8: 5′-CTCCTCGGTCATGTTGAGCATTTGG-3′; Sox9: 5′-GGGTCTAGGAGATTCATGCGAGAAA-3′ (described in Betancur et al., 2010, 2011); Sox10, 5′-CATGGTGACTTCCTTCTTCTCAATT- 3′ (described in Barembaum and Bronner, 2013), Sox5: 5′-CTTGGAAGACATCCTGGAAGGAACA-3′; Zic1, 5′-TGCGGTCCAGCATCCAGAAGCATCT-3′ (described in Simoes-Costa et al., 2012).

Comparative genomic and mutational analysis

Full length and deletion fragments of the Sox10E1 enhancer fragments was amplified from genomic chicken DNA using the Expand High Fidelity PCR System (Roche). The following primers were used: S10E1_S: GGAAGAGAGAAAGACCATGGTG; S10E1_S2: CATTCTCCAGTGGAAGGGGAC; S10E1_S3: CAGCATCCTTCCCTATCCCT; S10E1_S4: ATGGCGGACGCCAAAACG; S10E1_S5: GAGAAGGCTGAAGGCCACAG; S10E1_AS: AGTTGAATGGGTCCCTGG; S10E1_AS2: TTTGGCGTCCGCCATGGA; S10E1_AS3: TCCCCAGCCTTGCATCTGTAT; S10E1_AS4: CTTGGATGAGAGGAGGCGTC; Potential transcription factor binding sites were identified through the JASPAR (http://jaspar.genereg.net/) and TRANSFAC (http://www.gene-regulation.com/) databases. Using the USCS genome browser, predicted sites were screened for conservation across species. Selected sites were mutated by Fusion PCR (Szewczyk et al., 2006) using the following primers (mutated nucleotides are underlined and in bold): cMyb (M1): Fwd 5′-GTACGTACCCTTC**CTACAACAGC**CAAACAAAG-3′; Rev 5′-CTGCTTTCTTTGTTTG**GCTGTTGTAG**GAAGGGTAC-3′; FoxD3 (M2): Fwd 5′-**CTCACACAGC**CAGGTGGGCTCCATAT-3′; Rev 5′-**GCTGTGTGAG**TTTGAAGAACTGGAGAAGGG-3′; Zic (M3): Fwd 5′-G**CTACAACAGCCTACA**TGGCCCAGCATC-3′; Rev 5′-CA**TGTAGGCTGTTGTAG**CGAGAGGATTGC-3′; SoxD/E (M4): Fwd 5′-

CATCCTACACAGCCATTTAAAAAAAAAGGAGG-3′; Rev 5′-**GTGTAGGTAG**CTGCCCGCCTCC-3′; FoxD3 (M5): Fwd 5′-CTT**CTCACACAGC**GACAAGAAAACGACCCCC-3′; Rev 5′-TGTCC**GCTGTGTGAG**TTCCTCCTTTTTTTTTAAATGGCTT-3′; SoxE (M6): Fwd 5′-TGCTA**CTACCTACAC**CACCATTTCAGGGCTGAA-3′; Rev 5′-AATGGTG**GTGTAGGTA**CAATCGTTTTGGCGTCC-3′; Arid3A (M7): Fwd 5′-TGCAC**GCCGGG**GGATTCCACAGAGGGC-3′; Rev 5′-GTGGAATCC**CCCGGC**CACGTTGGTGTGGTGAAA-3′; Oct1 (M8): Fwd 5′-**CTCCCGGTT**CCTTTTCTTGGTAAGGATGG-3′; Rev 5′-**AACCGGGAG**AGCACTGTGGCCTT-3′; Elk/Ets (M9): Fwd 5′-**CTCCAGGAGC**CTTGTGTTCCTTGGGGTCAAC-3′; Rev 5′-**CTCCAGGAGC**CTTGTGTTCCTTGGGGTCAAC-3′; Amplified fragments were purified using the Wizard Gel and PCR extraction kit (Promega) and cloned into Asp780I/XhoI digested pTK GFP reporter vector (Uchikawa et al., 2003). For coelectroporation experiments, full length S10E1 was also subcloned into pTK mCherry vector. Deleted/mutated S10E1 sequences (in GFP) were coelectroporated with full length Sox10E1 (in mCherry) to control for electroporation efficiency.

Fluorescence Quantification

To quantify fluorescence intensity in electroporated embryos, we used ImageJ to measure the integrated density in the region of interest. To correct for different exposure levels between channels and individual embryos, we also calculated the integrated density from the mean of 3 background areas. The following formula was used to estimate the corrected total cell fluorescence (CTCF): Integrated density- (Area of selection x mean fluorescence of background readings). To adjust for variations in electroporation efficiency, fluorescence of WT Sox10E1 mCherry signals was normalized to morpholino FITC intensity. For mutated Sox10E1 constructs, GFP signal intensity of mutants was normalized to fluorescence intensity of WT Sox10E1 Cherry signal. At least 3 embryos were measured for every experiment. Statistical significance was estimated using unpaired t-test.

Generation of FLAG tagged Sox8

Chicken Sox8 was PCR amplified from cDNA using Phusion High Fidelity Polymerase system (New England Biolabs). The FLAG epitope was added to the 3′ end of the coding sequence and the sequence was ligated into XhoI/NheI digested pCI-H2B-GFP vector and confirmed by sequencing. The following primers were used: Fwd: 5′-ATGCTCAACATGACCGAGGA-3′, FLAG_Rev: 5′-TTACTTGTCATCGTCGTCCTTGTAGTCAGGCCTCGTCAGGGTTGT-3′;

Probe preparation and In situ hybridization

RNA *in situ* hybridization on whole mount chicken embryos was performed as described (Henrique et al., 1995) with slight modifications of the post hybridization washes. Dioxigenin labeled antisense probes were generated by *in vitro* transcription from linearized templates using Promega RNA polymerases. Probes used were: Sox10 (Betancur et al, 2010), Sox8 (Betancur et al, 2011), Sox5. The template for the Sox5 probe was generated by

PCR amplification from the chicken EST clone ChEST277j14 using the following primers: Fwd: 5′-ATGCTCACTGACCCTGATTTAC-3′; Rev: 5′-TGGCCGAAGGACTAGCTAAT-3′. Bound probes were detected using an Anti-Dig antibody conjugated with alkaline Phospatase and NBT/BCIP as the color substrate.

Immunohistochemistry

Embryos were fixed 20 minutes with 4%PFA and washed with TBS containing 0.3%Triton, 1% DMSO. Unspecific binding was blocked with donkey serum and primary antibody incubation was carried out overnight at 4°C. The antibody used was anti human Sox10 (R&D systems AF2864). Signals were visualized with an Alexa 594 conjugated anti goat antibody (ThermoFisher).

Immunoprecipitation of protein complexes

Trunk neural tubes of electroporated embryos were dissected in ice cold PBS and homogenized in IP lysis buffer (50mM Tris pH 7.4, 150mM NaCl, 1% NP40). After pre clearing with unconjugated beads, lysates were distributed to antibody coated G-agarose beads (Sigma Aldrich) and incubated at RT for 2–3 hours. Samples were washed 6 times with IP lysis buffer before precipitated proteins were eluted from beads and subjected to SDS page. Ten microgram of the following antibodies were used for immunoprecipitation reactions: anti Sox5 (Abcam ab94396, rabbit, ChIP grade), anti rabbit control IgG (Abcam ab46548, ChIP grade), anti FLAG M2 (Sigma-Aldrich, F1804, mouse IgG1), anti mouse IgG1 (Santa Cruz, sc-3877).

Chromatin Immunoprecipitation (ChIP)

Trunk neural tubes of stage HH14-16 embryos were dissected in ice cold PBS and homogenized in isotonic buffer (10mM Tris pH 7.5, 3mM $CaCl_2$, 0.25M Sucrose, 0.5% Triton, 1mM DTT, supplemented with Protease inhibitor cocktail, PMSF and Sodium butyrate). Chromatin was subsequently cross-linked by addition of formaldehyde to a final concentration of 1%. Cross-linking was stopped by addition of 125mM glycine. The chromatin isolation procedure was performed as previously described (Hauser et al., 2002). For ChIP, equal amounts of sonicated chromatin were diluted 10-fold and precipitated overnight with the following antibodies: anti Sox5 (Abcam ab94396, rabbit, ChIP grade), anti rabbit control IgG (Abcam ab46548, ChIP grade). Chromatin–antibody complexes were isolated with protein A magnetic beads (Dynabeads, Invitrogen). Precipitated DNA was analyzed by real time PCR using SYBR Green (Bio-Rad) on an ABI7000 qPCR machine. Diluted Inputs were used as Standards.

The following primers were used: 10E1_M4_S1 5′-CGCTGGTAACAGAGGGGTTA-3′; 10E1_M4_AS1 5′-GGGGTCGTTTTCTTGTCCTT-3′; 10E1_M4_S2 5′-GCATCCTTCCCTATCCCTTT-3′; 10E1_M4_AS2 5′-AAATGGTGCCTTTGTGCAAT-3′; 10E1_M4_S3 5′-TGGTGTGGGTGAACAGAAGA-3′; 10E1_M4_AS3 5′-TTCTACTTGTGGGGGCACTC-3′; 10E1_M4_S4 5′-CAGGGAACAAAGAAGCCATT-3′; 10E1_M4_AS4 5′-AATCACGTTGGTGTGGTGAA-3′; Control_S 5′-GGTTGGATTTCCAGTCTCCA-3′; Control_AS 5′-TGTTTTGCTGGACAATCTGC -3′.

Western Blotting

Protein lysates were separated by SDS PAGE and subsequently transferred onto nitrocellulose membrane. After blocking, membranes were incubated with the primary antibody overnight, followed by washes in PBST and incubation with HRP conjugated secondary antibodies (KPL). Signals were visualized using Amersham prime western blotting detection reagent (GE Healthcare). The following primary antibodies were used: Anti Sox5 (Abcam ab94396 rabbit), Anti Tubulin (Sigma-Aldrich T9026, mouse IgG), Anti FLAG M2 (Sigma-Aldrich F1804, mouse IgG1).

Imaging and Figure Preparation

Whole mount embryos and sections were imaged on a Zeiss fluorescent Axioimager D2 research microscope equipped with an Apotome2 and dual Axiocam 506 cameras. Pictures were taken with Zen2pro Software. A Zeiss axioskop2 microscope equipped with Axio Vision software was used to image *in situ* stained embryos. Images were processed using Adobe Photoshop CC15 and figures were prepared in Adobe Illustrator CC15.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank M. Barembaum and M. Simões-Costa for plasmids and morpholinos. This work was supported by grants DE024157 and HD037105 to MEB and a postdoctoral fellowship from the Curci foundation and an Erwin Schrödinger fellowship from the Austrian science fund FWF (J3538-B19) to CM. The authors declare no competing financial interests.

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Highlights

- The chicken Sox10E1 enhancer is active in migrating trunk neural crest and the otic vesicle
- Two Sox binding sites are critical for enhancer activity in both tissues
- Knockdown of Sox8 and Sox5 reduces neural crest specific Sox10E1 reporter activity
- Sox5 occupies the Sox10E1 enhancer in the trunk
- Sox5 and Sox8 can physically interact with each other

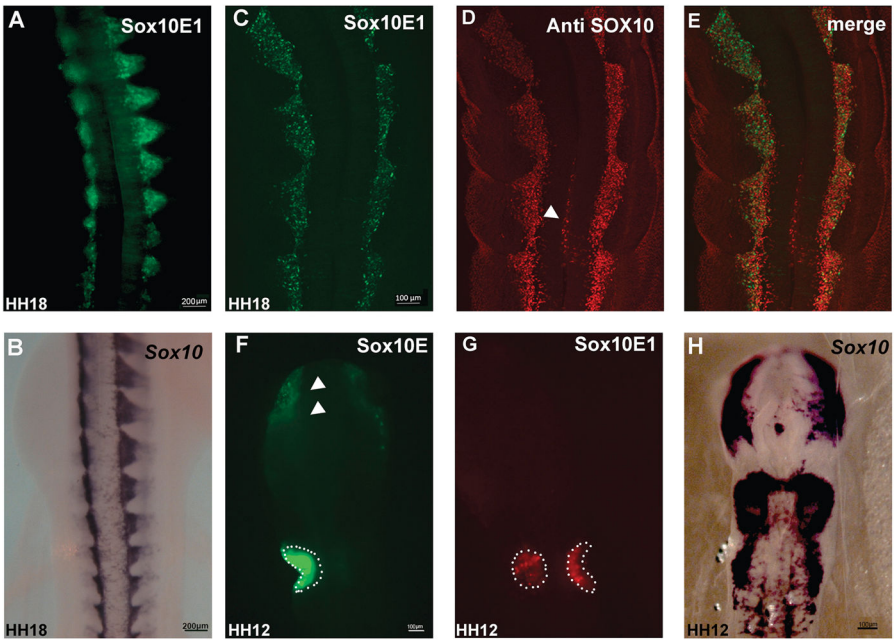

Figure 1. Activity of the Sox10 E1 enhancer

EGFP expression driven under control of the Sox10E1 enhancer fragment in stage HH18 chick embryos (A, C). Endogenous Sox10 expression pattern at the corresponding developmental stage: *In situ* hybridization to detect Sox10mRNA levels (B) and antibody staining (D) on electroporated embryos (merge in E). Note that while endogenous Sox10 protein is also expressed in the delaminating NC cells (arrowhead in D), the GFP signal is only visible in the migratory cells that have detached from the neural tube. Comparison of Sox10E-GFP (including Sox10E2, F) with Sox10E1-mCherry activity (G) and endogenous Sox10 expression levels (H, *in situ*) at stage HH12. Sox10E1 is restricted to the otic placode at that stage and not active in the cranial neural crest (arrowheads in F).

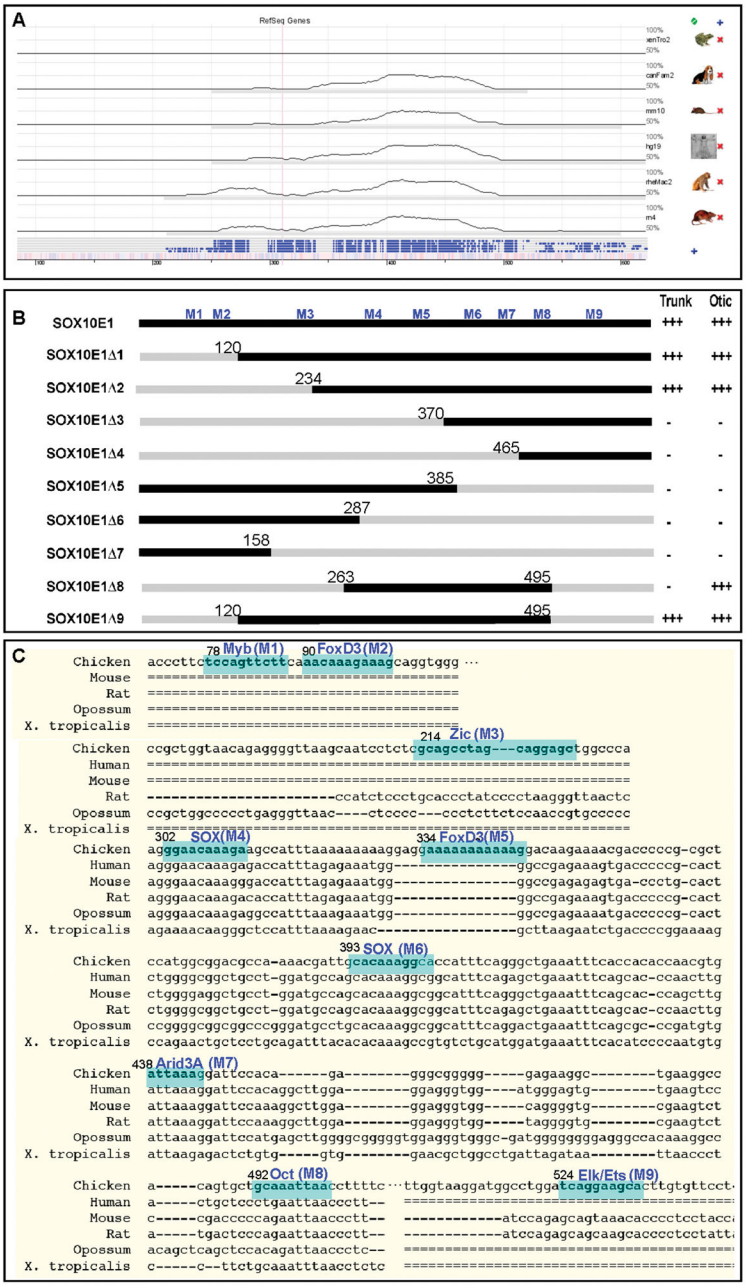

Figure 2. Sox10E1 sequence conservation and analysis

Output of the ECR genome browser showing the sequence conservation of chicken Sox10E1 throughout different species (A). The element is found on chromosome 1, position 53010774-53011395. Summary of the deletion constructs generated in this study is shown in (B). Corresponding positions of the mutations are indicated. Conservation across species was analyzed using the UCSC genome browser (C).Mutated sites are indicated.

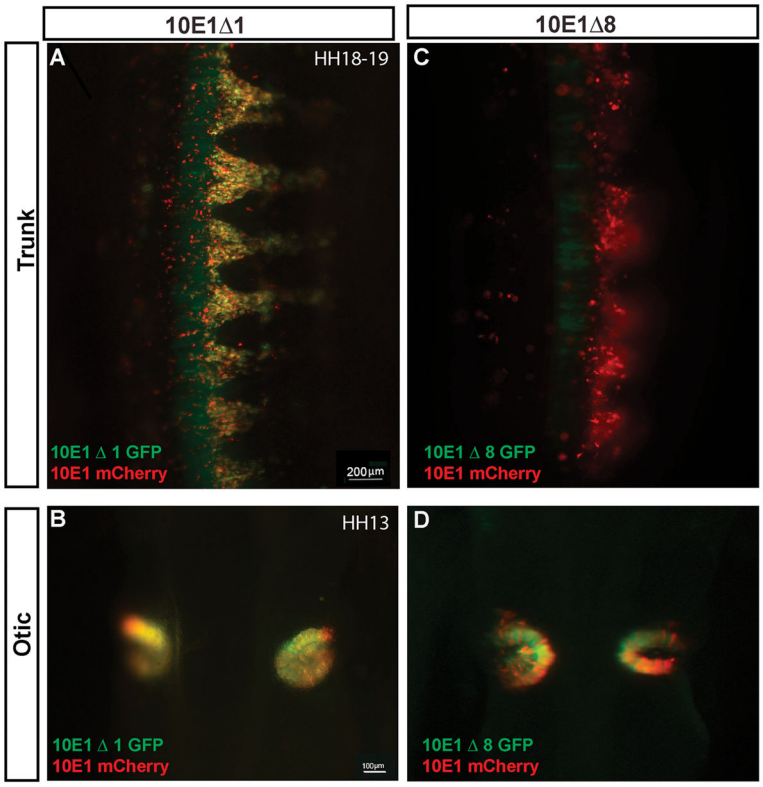

Figure 3. Defining essential regions mediating trunk expression

Serial deletions of the Sox10E1 fragment were tested for their ability to drive GFP *in ovo.* While So10E1Δ1 still drives robust GFP expression in the trunk neural crest (A) and in the otic vesicle (B), Sox10E1Δ8 has lost trunk neural crest activity (C) while still being active in the otic vesicle (D). In all cases, the deleted version was coinjected with the full length Sox10E1mCherry. Merged images are shown.

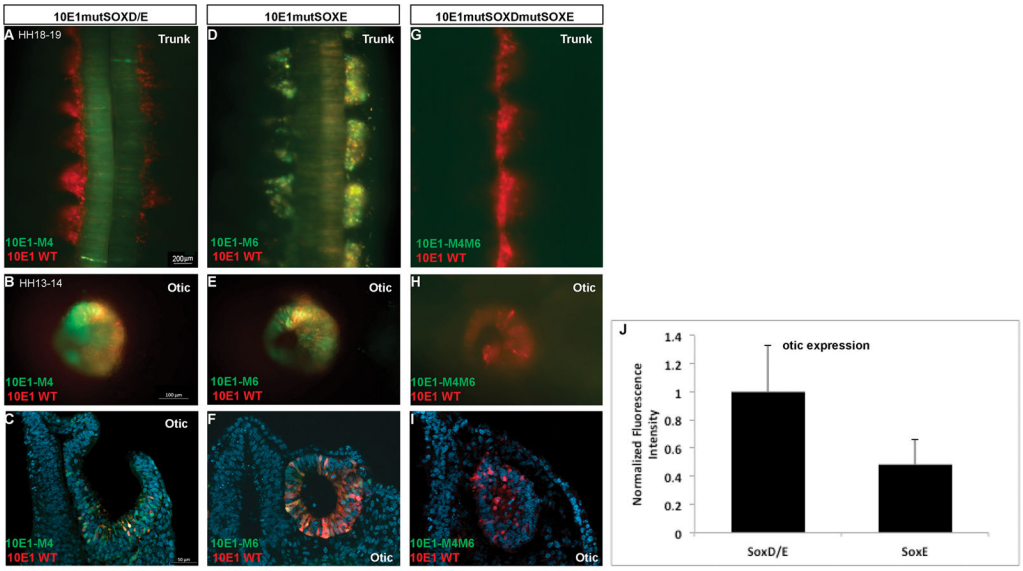

Figure 4. Two SOX sites have differential regulatory function

Mutation of a potential SoxD/E binding site (M4) omits expression in the trunk (A), but is still active in the otic vesicle (B–C). Mutating a putative SoxE binding site (M6) has no effect on trunk expression (D) but reduces expression in the otic vesicle (E–F). Mutating both sites completely abolishes expression in both tissues (G–I). In all cases, mutant constructs are marked by GFP and co electroporated with the wild type version of the Sox10E1 enhancer tagged with mCherry. C, F and I show representative sections through the otic vesicle. Merged images are shown. Quantified otic fluorescence for M4 and M6 are shown in (J). P= 0.06.

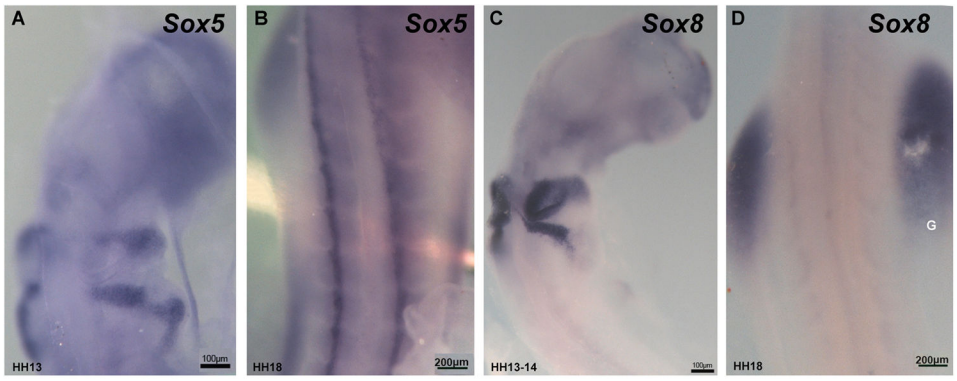

Figure 5. Expression of Sox5 and Sox8 during stages of Sox10E1 enhancer activity

In situ hybridization showing expression of Sox5 during vagal and trunk neural crest migration (AB). Sox5 is strongly expressed in the migratory crest cells streams surrounding the otic vesicle but not in the otic vesicle itself (A). Later on, it is present in the trunk neural crest, somites and surrounding mesoderm (B). *In situ* hybridization for *Sox8* shows expression in the otic vesicle and in the R6 stream at HH13 (C). During trunk neural crest migration, Sox8 is expressed at lower levels in the migratory crest (D).

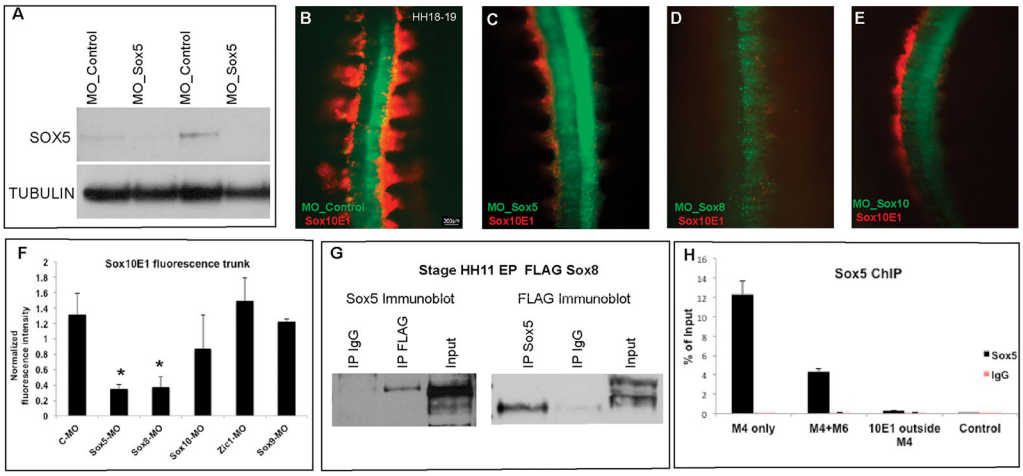

Figure 6. Sox5 and Sox8 regulate trunk Sox10E1 enhancer activity

Knockdown efficiency of the Sox5 morpholino was confirmed by Western blot (A). Samples were taken from stage HH4 electroporated embryos. Tubulin was used as loading control. Co electroporation of Sox10E1 (in red, Cherry) with a control morpholino (in green, FITC) in the trunk exhibits strong mCherry signal in the migratory crest cells (B). In contrast, Sox10E1 activity (as measured by mCherry signal intensity) is significantly weaker compared to the morpholino signal in the presence of a Sox5 morpholino (C). A similar reduction in Sox10E1 expression is observed upon knockdown of Sox8 (D). Sox10 morphant embryos partially exhibit a reduction in Sox10E1 expression (E). Merged images are shown. Summary and quantification of fluorescence intensity is shown in (F). ***** indicate $P < 0.05$. Western blots from pull downs of FLAG Sox8 electroporated embryos (G). The immunoprecipitation reaction was carried out with either FLAG (blot 1) or Sox5 (blot 2) antibody, and the respectively other antibody was used for the immunoblot detection. ChIP assay was performed on trunk regions of WT chick embryos (H). Sox5 is specifically bound to the M4 binding site.

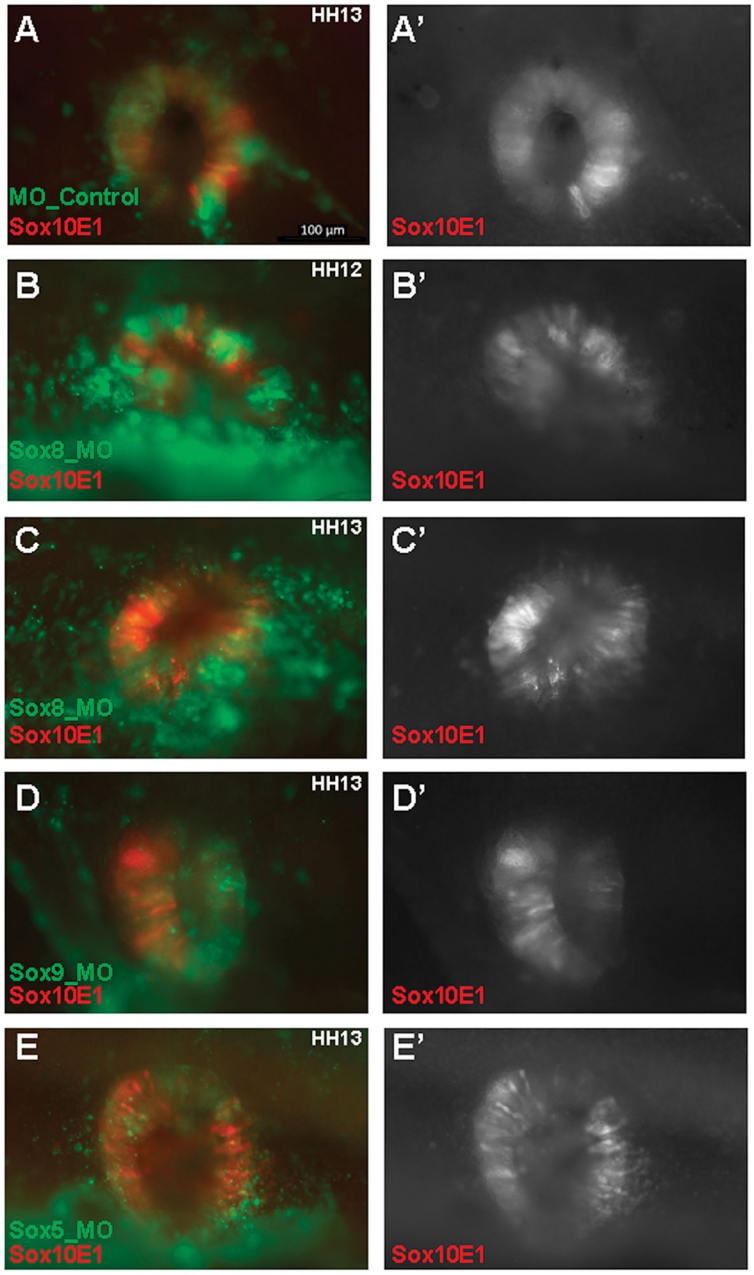

Figure 7. Sox8 is involved in regulating otic Sox10E1 activity

Co-electroporation of Sox10E1 (red, Cherry) with a control morpholino (green, FITC) leads to robust reporter expression in the otic vesicle (A–A′). Knockdown of Sox8 exhibits stage specific effects on reporter expression (B, C). While a knockdown at stage HH8 or earlier reduces reporter expression (B–B′), a knockdown of HH9 has only mild effects on Sox10E1 activity (C–C′). Knockdown of Sox9 in the otic vesicle (D–D′). Some embryos exhibit a reduction at early stages. Sox5 knockdowns do not effect Sox10E1 expression (E–E′). Merged images and individual channels of Sox10E1 reporter are shown.

Table 1

Summary of mutations tested in this study.

Mutation	Expression Trunk	Expression Otic
M1:Myb	+++	+++
M2:FoxD3	+++	+++
M3: Zic	+++	+++
M4: SOXD/E	−	+++
M5: FoxD3	+++	+++
M6:SOXE	+++	+−
M7: Arid3A	+++	**+ ++**
M8: Elk/Ets	+++	+++
M9: Oct	+++	+++
M4M6: SOXD/E+ SOXE	−	−

+++ indicate strong activity,

+− indicate reduced activity,

− indicate no activity